INVESTIGATION ON THE MUTAGENICITY OF *N*-NITROSOTHIAZOLIDINE USING THE AMES *SALMONELLA* TEST

INTRODUCTION

Two research groups, including our own, have recently identified *N*-nitrosothiazolidine (NTHZ) in bacon (Gray *et al.*, 1982; Kimoto *et al.*, 1982). Since the original identification of this compound, we have developed a dual-column chromatographic method for its simultaneous determination with *N*-nitrosodimethylamine and *N*-nitrosopyrrolidine (Pensabene & Fiddler, 1982) and conducted studies demonstrating that NTHZ is present in bacon as a result of smokehouse processing (Pensabene & Fiddler, 1983a).

Little is known about the toxicity of NTHZ, although this *N*-nitrosamine, formed from the Maillard-Browning reaction and nitrite, was found to be a direct-acting mutagen with *Salmonella typhimurium* TA100, the activity of which was suppressed by a 9 000 × g supernatant of rat liver homogenate (Mihara & Shibamoto, 1980; Sekizawa & Shibamoto, 1980).

As an initial toxicological evaluation, we carried out the Ames *Salmonella* test on NTHZ to verify the mutagenicity of this compound. Our findings are presented here.

MATERIALS AND METHODS

Materials

Cysteamine hydrochloride, thiazolidine, formaldehyde (37%), sodium nitrite and all other reagents were purchased from commercial suppliers and used without further purification.

N-Nitrosothiazolidine synthesis

Thiazolidine method: Sodium nitrite (0.30 mol) was added slowly to a cooled (0 °C), stirred, aqueous solution of thiazolidine (0.28 mol) and 4.5 mol/L sulfuric acid (0.28 mol). After addition, the cold mixture was stirred for an hour, then for another hour at room temperature. The reaction mixture was extracted with dichloromethane (DCM), the DCM was washed with 30% potassium hydroxide, dried over anhydrous sodium sulfate and concentrated on a rotary evaporator. The oily residue was vacuum distilled to obtain NTHZ (85 °C at 0.55 torr).

Cysteamine/formaldehyde method: An aqueous solution of cysteamine hydrochloride (0.18 mol) and formaldehyde (0.18 mol) was stirred at room temperature for 24 h. This solution was cooled to 0 °C and 4.5 mol/L sulfuric acid (0.18 mol) were added. Sodium nitrite (0.20 mol) was then added slowly and the mixture stirred at 0 °C for one hour, then at room temperature for another hour. NTHZ was isolated as described by Ray (1978), except that DCM was used instead of ether to extract the nitrosamine from the reaction mixture.
Caution: Nitrosamines are potential carcinogens. Exercise care in handling these materials.

N-*Nitrosothiazolidine confirmation*

N-Nitrosothiazolidine from both sources was confirmed by gas-liquid chromatography-mass spectrometry (GLC-MS) (Hewlett Packard, model 5992B), with no apparent difference in their spectra when these were obtained under conditions described previously (Kimoto *et al.*, 1982). No difference was observed in the spectra obtained from the NTHZs (10 µL/10 mL hexane) when scanned from 260 to 400 nm on a Hewlett-Packard Model 8450A UV/VIS spectrophotometer. Four major peaks were noted: λ_{max} 283, 360, 372, and 386. Both samples of NTHZ were more than 99% pure, as indicated by GLC, using a 1.8 m × 2 mm column containing 15% Carbowax 20M-TPA on 60/80 Gas Chrom P.

Mutagenicity assay

Mutagenicity assays were performed in accordance with the preincubation method described by Kitamura *et al.* (1981). *Salmonella typhimurium* TA100 (a gift of Dr B.N.Ames) was used as the indicator organism (Ames *et al.*, 1975). Approximately 2×10^8 cells were placed in sterile test tubes to which were added 100 µL dimethylsulfoxide, alone or with the dissolved test compound, and 500 µL phosphate buffer (0.1 mol/L, pH 7.4).The solutions were mixed and incubated for 20 min at 37 °C. At termination, 2 mL of 50 °C soft top agar (0.5%) were added to the incubating solutions. They were then mixed and poured onto minimal medium in petri dishes (Miller & Buchanan, 1983). Appropriate solvent and known mutagen (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 10µg/plate) controls were included each day with the experimental samples. Genetic markers were checked routinely according to the method of Ames *et al.* (1975). After an hour, the dried plates were inverted and incubated for 48 h at 37 °C. *His*$^+$ revertant colonies were counted using a Biotran automated colony counter (New Brunswick Scientific Co.). All experiments were performed at least twice, using triplicate plates per determination. A positive mutagenic response was defined as a minimum of twice the solvent-control reversion yield.

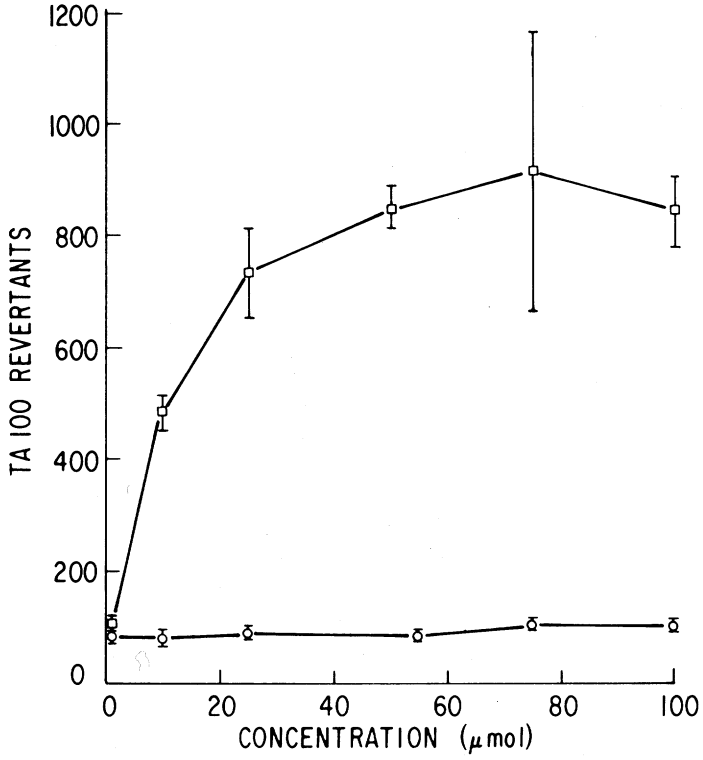
High-performance liquid chromatography (HPLC) analysis

Fifty µL of NTHZ distillate, obtained from cysteamine/formaldehyde/nitrite, were diluted with 50 µL hexane-DCM (1:1, v/v) and separated by HPLC (Milton Roy) using a 3.2 mm × 25 cm, 5 µ Spherisorb silica column and a variable-wavelength detector (Perkin-Elmer, Model 55) set at 365 nm, the maximum absorbance of NTHZ. A linear solvent gradient of 100% hexane to 100% DCM over a 15-min, interval was employed, with a flow rate of 1 mL/min. Sample fractions were collected after 2 min, for 4- or 6-min intervals, up to 30 min.

RESULTS AND DISCUSSION

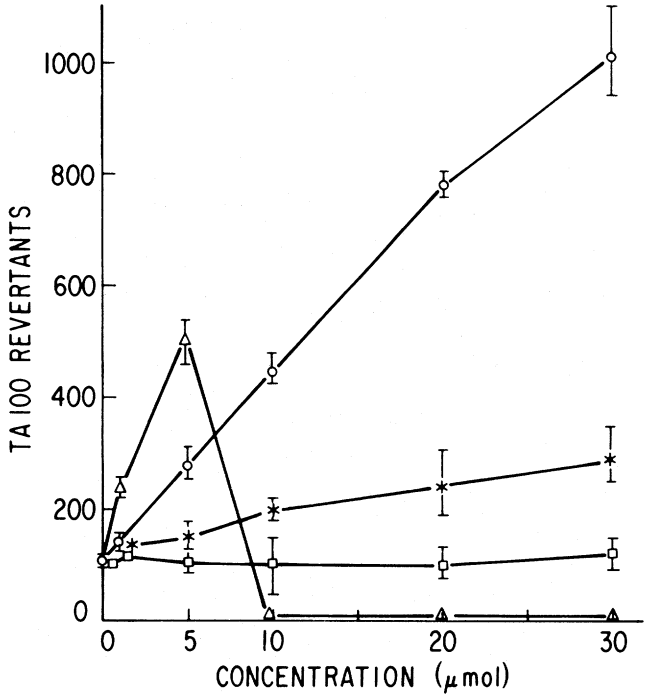
N-Nitrosothiazolidine, synthesized by direct nitrosation of thiazolidine, was tested for mutagenicity by the Ames assay procedure. This compound was shown not to be mutagenic to TA100 over a 2 log dose range. However, when NTHZ was synthesized from cysteamine/

Fig. 1. Mutagenic activity of *N*-nitrosothiazolidine synthesized by two different procedures: □, cysteamine/formaldehyde/nitrite; o, thiazolidine/nitrite



formaldehyde/nitrite according to the procedure of Mihara and Shibamoto (1980), the product was found to be mutagenic (maximum response was approximately nine times the spontaneous level). The mutagenic responses of NTHZ synthesized by the two different methods are shown in Figure 1. It is likely that the other investigators did not detect mutagenicity of NTHZ, but of some other compound. Our results suggest that the mutagenic species obtained by the cysteamine/formaldehyde/nitrite reaction, which we henceforth designate 'NTHZ', was either a trace contaminant, formed as a result of a side reaction, or a residual reaction precursor. For this reason, we tested formaldehyde, cysteamine, thiazolidine and nitrite individually for potential genotoxic activity, using the same conditions as for 'NTHZ'. The results for all compounds except nitrite are shown in Figure 2. Formaldehyde was strongly mutagenic at the 5-μmol level, but became cytotoxic at higher doses; Donovan *et al.* (1983) made a similar observation. However, it was thought that formaldehyde was not the mutagenic species observed in 'NTHZ', since its presence was unlikely, due to its volatility and high reactivity. Cysteamine was found not to be mutagenic over a 0-30-μmol dose range. This compound has, in fact, been shown to offer protection against genotoxic agents by what is thought to be radical scavenging activity (Bianchi *et al.*, 1982). Thiazolidine was tested because its mutagenic activity was reported by Mihara and Shibamoto (1980): we observed moderate mutagenicity at concentrations above 20 μmol/plate, which is not sufficient to account for the mutagenicity of 'NTHZ'. The results for nitrite, not shown on the figure, gave no evidence of mutagenic activity

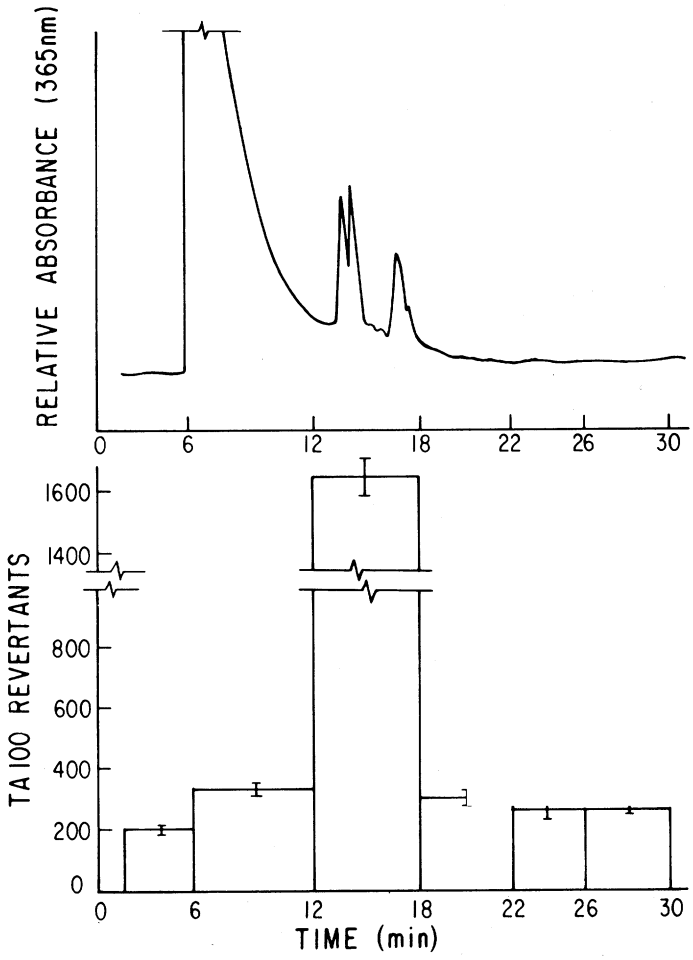
Fig. 2. Mutagenic activity of '*N*-nitrosothiazolidine and its precursors: ○, *N*-nitrosothiazolidine; *, thiazolidine; □, cysteamine; △ formaldehyde



under the conditions employed, which involved the use of a buffer at pH 7.4. If the test had been run under acidic conditions, nitrite, or, more precisely, nitrous acid, would be expected to be mutagenic, since it is known to deaminate nucleotides such as adenine (Nicholson-Guthrie, 1970). Generally, these results and those shown in Figure 2 suggest that the genotoxic response observed with 'NTHZ' was not due to residual precursors.

Studies were then carried out to separate the 'NTHZ' by HPLC (using a silica column) to determine if a mutagenic contaminant was present. The chromatograph is shown in Figure 3. The fraction collected between 6 and 12 min, independently determined to contain NTHZ, exhibited no mutagenic activity, whereas, the fraction collected between 12 and 18 min (containing several small peaks) showed strong mutagenic potential, as indicated in the same figure. The other fractions were found to contain no mutagenic activity. These data suggest that the mutagenic activity observed with 'NTHZ' was due to one or more contaminants resulting from the cysteamine /formaldehyde/nitrite reaction. It should be noted that the contaminant(s) is probably present in a very low concentration and, given the relatively strong mutagenic response, would have to be considered a potent directly-acting mutagen. Most *N*-nitrosamines, but not nitroso-amides and ureas, require metabolic activation before they are mutagenic. The latter classes of compounds are potent directly acting mutagens and animal carcinogens and have been implicated epidemiologically in human gastric and oesophageal cancer (Mirvish, 1971; Correa *et al.*, 1975). Generally there is a good correlation between in-vitro mutagenicity and rodent carcinogenicity for promutagenic nitrosamines if the liquid preincubation technique is used, as described by Yahagi *et al.* (1977), yet some carcinogenic nitrosamines have been reported to be non-mutagenic and *vice versa* (Rao *et al.*, 1979).

Fig. 3. '*N*-nitrosothiazolidine': high-performance liquid chromatogram and mutagenic response of six sample fractions



Investigators have sugggested that the discrepancies result from inappropriate in-vitro simulation of the in-vivo transformation that is necessary to form the active metabolite.

While the results of this study indicate that NTHZ is not mutagenic in our test system, the *N*-nitrosamine and the contaminant are still of toxicological concern. The fact that a mutagenic contaminant is formed during the synthesis of NTHZ by the cysteamine/formaldehyde/nitrite reaction and that NTHZ is present in bacon and other cured-meat products (Pensabene & Fiddler, 1983b), suggests the possibility that the mutagenic contaminant is also present in the latter. If the mutagen is found not to be a *N*-nitrosamine, it is possible that the Browning reaction, which forms thiazolidines from cysteamine and glucose (Kitamura *et al.*, 1981), can also form the mutagen or its precursor during the processing and/or cooking of cured meats. For this reason, it is important to identify the mutagen in 'NTHZ', determine how it is formed and develop means for its total or partial elimination. Research is currently in progress in these areas.

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